Nucleic Acids Research, Vol. 20, No. 4 735–745

Molecular cloning of the cDNA for the catalytic subunit of human DNA polymerase δ

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Received November 25, 1991; Revised and Accepted January 16, 1992 GenBank accession no. M81735

ABSTRACT

The cDNA of human DNA polymerase δ was cloned. The cDNA had a length of 3.5 kb and encoded a protein of 1107 amino acid residues with a calculated molecular mass of 124 kDa. Northern blot analysis showed that the cDNA hybridized to a mRNA of 3.4 kb. Monoclonal and polyclonal antibodies to the C-terminal 20 residues specifically immunoblotted the human pol δ catalytic polypeptide. A multiple sequence alignment was constructed. This showed that human pol δ is closely related to yeast pol δ and the herpes virus DNA polymerases. The levels of pol δ message were found to be induced concomitantly with DNA pol δ activity and DNA synthesis in serum restimulated proliferating IMR90 cultured cells. The human pol δ gene was localized to chromosome 19 by Southern blotting of EcoRI digested DNA from a panel of rodent/human cell hybrids.

INTRODUCTION

DNA polymerase δ (pol δ) was discovered as a new type of DNA polymerase which at the time was unique among mammalian DNA polymerases in possessing an intrinsic 3′ to 5′ exonuclease activity (1, for reviews see 2–4). Pol δ has been isolated from rabbit reticulocytes (1), calf thymus (5–7) and human placenta (8). Purified calf thymus pol δ has subunit polypeptides of 125 and 48 kDa (7); the 125 kDa subunit of human pol δ has been demonstrated to be the catalytic polypeptide (8). Pol δ has been shown to play a critical role in eukaryotic DNA replication. Studies in permeabilized cell systems have implicated pol δ activity in both DNA replication and repair functions (9–11). A 36 kDa factor, first identified in calf thymus (7), was shown to convert pol δ activity from low to high processivity when acting on sparsely primed templates (12). This factor was subsequently shown to be Proliferating Cell Nuclear Antigen (PCNA) (13), whose expression is strongly tied to the S phase of the cell cycle. Most significantly, both pol δ and PCNA are required for the *in vitro* replication of the SV40 chromosome (14, 15). Current models of the eukaryotic replication fork involve the participation of both pol α and δ, with these having primary functions at the lagging and leading strands, respectively (2, 16, 17). A second form of DNA polymerase δ in mammalian cells was subsequently discovered, based on the possession of a larger catalytic polypeptide with reported molecular weights ranging from 140 to 215 kDa (18–22). This form of DNA polymerase δ was distinguished from the 125 kDa form by a relative insensitivity to PCNA when polyd(A)/oligo(dT) was used as a template, and was later termed pol ε (23).

Studies of yeast DNA polymerases I, II and III have suggested that they represent a complement of enzymes analogous to those of mammalian cells. For example, yeast pol δ, the product of the *S. cerevisiae CDC2* gene, appears to be the homolog of the 125 kDa form of mammalian pol δ, based on the similarities in enzymatic properties, which include a response to PCNA, the possession of an intrinsic 3′ to 5′ exonuclease activity and a molecular mass of 125 kDa (4, 24). Thus, a common nomenclature of the yeast mammalian enzymes as α, δ and ε, respectively, has been proposed (23). However, a structural similarity had only been demonstrated for yeast pol I and human pol α (25, 26). Yeast pol δ is also structurally related to the human herpes virus family of DNA polymerases (27). These findings have led to the concept that the mammalian and lower eukaryotic DNA polymerases may be closely conserved (28).

There has been a rapid and dramatic increase in our knowledge of the DNA polymerases at the structural level for the yeast, viral and bacteriophage DNA polymerases. Sequence comparisons of human pol α have shown that it is a member of a protein family of diverse evolutionary origins, including human viruses and bacteriophages (28). Conversely, E. coli pol I and a number of other prokaryotic DNA polymerases represent a separate family. However, there is currently only indirect evidence for structural relationships between mammalian pol α, δ and ε (8) since human pol α (25) is the only member of this group that has been cloned. We report here the cloning of the cDNA for human pol δ, a comparison of its primary structure with those of other DNA polymerases, as well as studies on its expression and chromosomal localization.

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MATERIALS AND METHODS

Materials

A HeLa cell cDNA library constructed in λgt10 was a generous gift from Drs. Craig Hauser and Robert Tijian, University of California at Berkeley; a random-primed human fibroblast cDNA library was a generous gift from Dr. Frederick S. Hagen of Zymogenetics Inc. Human placental DNA polymerases α, δ and ε were prepared as previously described (8). PCR amplifications were performed using a thermal cycler and reagents were obtained from the Perkin-Cetus Corp. Nested primers for the 'RACE' primer-extension protocol (29, 30) were generous gifts from Dr. Michael A. Frohman, University of California at San Francisco. Human/rodent somatic cell hybrid mapping panel #2, consisting of DNA isolated from 24 human/rodent somatic cell hybrids retaining one or two human chromosomes, was obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ).

PCR amplification of mRNA

The existence of highly conserved regions in the family of DNA polymerases which includes human pol α and yeast pol δ (25, 27) was the basis for the generation of a series of primers for PCR amplification of human polyA$^+$ mRNA. Antisense primers based on the sequence of region I, the most highly conserved region, were used to prime HeLa mRNA using AMV reverse transcriptase (BRL). A series of 'second primers' with limited degeneracies were designed (31). These were based on sequences from conserved regions IV, II, VI and III of human pol α (25) and yeast pol δ (27). After the first-strand cDNA synthesis, the second primer was added and PCR amplification was performed. The PCR products were examined by agarose gel electrophoresis, and selected for further analysis on the basis of correlations of size with predicted length, assuming conservation of the spatial separation of conserved domains in human and yeast pol δ. Putative positive PCR products were subcloned into a sequencing vector [pGEM3z or pBluescript II KS(+)] and partially sequenced for the first 200−300 bp at the 5′ and 3′ ends to permit comparison of their open reading frames with human pol α and yeast pol δ sequences. Alternatively, PCR products were directly sequenced (32). After a number of trial experiments we isolated a clone (PCR-1) containing an open reading frame which was highly similar to yeast DNA pol δ (see RESULTS). This was isolated using the partially degenerate primers [GAT/CCCA/TG-AT/CGTT/G/CATA/T/CATA/TGGA/G/C/TTAT/CAA and G-AATTCTCA/G/C/TGTA/GTCA/G/C/TCCA/GTAA/G/C/T-AC/TA/G/C/TAC]. These were based on residues 392−400 (DPDVIIGYN) and residues 755−761 (VVYGDTD) in regions IV and I, respectively, of the yeast pol δ sequence (27).

Isolation of additional PCR clones by primer extension PCR

PCR primer extension amplification in the 5′ direction from mRNA transcripts was performed by the method of Frohman et al. (29), and by the 'RACE' method using linker primers (30) and a set of three antisense primers. PCR amplification from human cDNA libraries in λ phage was performed using λgt10 primers in combination with antisense primers (33).

Screening of human cDNA libraries

The human cDNA libraries in λgt10 and λgt11 phages were plated and screened with the randomly primed cDNA probes (34). Filters were hybridized to the ^{32}P-labeled probes in 50% formamide, 5×Denhardt's solution, 5×SSPE, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA at 42°C overnight. The filters were washed 3 times in 2×SSC, 0.1% SDS and once in 1×SSC, 0.1% SDS at 42°C, air-dried and exposed to Kodak XAR-5 film overnight. Positive clones were plaque purified and the phage DNA were isolated by discontinuous cesium chloride gradient ultracentrifugation.

Northern blotting

Total RNA was isolated by a single-step method by acid guanidinium thiocyanate-phenol-chloroform extraction (35). PolyA$^+$ mRNA was also selected by using oligo(dT)-cellulose columns. RNA (30 μg) was electrophoresed on 1.2% agarose/5% formaldehyde gels and transferred to nitrocellulose. The blots were hybridized overnight with randomly primed ^{32}P-labeled probes at 42°C in 50% formamide, 5×Denhardt's solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, 25 mM sodium phosphate pH 6.8, 5×SSC (0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0), and washed twice in 1×SSC, 0.1% SDS at room temperature for 1/2 hr and subsequently in 0.25×SSC, 0.1% SDS at 55°C for 1 hr and finally in 0.1×SSC at 55° C for 1 hr.

Sequencing strategy

Unidirectional deletion subclones of the cDNAs from both 5′ and 3′ ends were generated by digestion with exonuclease III or Bal31 nuclease (36). The cDNAs in each subclone were sequenced by the dideoxynucleotide chain termination method (37) using T7 DNA polymerase (Sequenase). 7-Deaza-dGTP or dITP were used in eliminating sequence compression in GC-rich regions of the

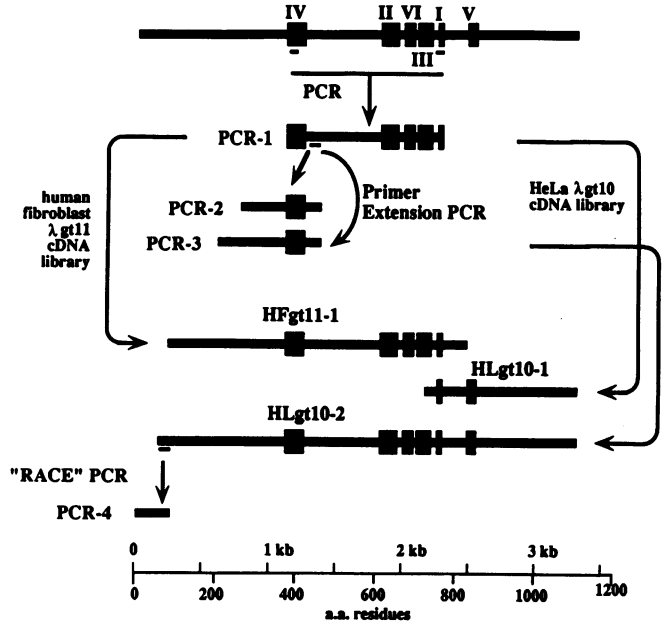

Fig. 1. Isolation of the cDNA for DNA polymerase d. The diagram shows schematically the progress of the isolation of the cDNA by a combination of PCR and conventional screening of cDNA libraries. The locations of the conserved regions I through VI (25) in the amino-acid sequence are marked by boxes. The DNA sequences of all the isolates shown above were determined by sequencing on both strands. The locations of the cDNA isolates in relation to the overall cDNA sequence are as follows: PCR-1 (nts 1207−2311); PCR-2 (nts 921−1465); PCR-3 (nts 892−1486); PCR-4 (nts 1−449); HFgt11-1 (nts 343−2517); HLgt10-1 (nts 2239−3502); HLgt10-2 (238−3502).

cDNA. Specific primers were also synthesized and used to sequence regions where insufficient overlaps were found or where ambiguities were encountered.

Preparation of antibodies

The C-terminal peptide (DLEDQEQLLRRFGPPGPEAW) was synthesized by the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainsville, FL. Polyclonal antibodies and monoclonal antibodies against this peptide were generated essentially as described previously (8). The peptide was covalently attached to cyanogen bromide-activated Sepharose (Pharmacia). The polyclonal antibody was then purified by affinity chromatography on the peptide-Sepharose column.

Amino acid sequence alignments

DNA polymerase sequences used for alignment were as follows: herpes simplex virus type 1 (38); Epstein-Barr virus (39); cytomegalovirus, (40); varicella zoster virus (41); yeast pol δ (27, as corrected in refs. 42, 43); yeast pol ε (44); yeast pol α (26); human pol α (25); T7 (45); *S. pombe* pol δ (46); REV3 (47); T4 (48); E. coli pol II (49). Pairwise alignments were performed with the ALIGN program of the Protein Identification Resource, National Biomedical Research Foundation, Washington DC. Multiple sequence alignments were performed by compilation of the pairwise alignments followed by visual adjustment. The MATCH and FASTP programs of the PIR were also used for database searches for conserved motifs.

RESULTS

Isolation and nucleotide sequence of the cDNA of human DNA polymerase δ

A positive PCR product (PCR-1) of 1.1 kb was isolated by PCR amplification of HeLa mRNA using primers based on the sequences VVYGDTD and DPDVIIGYN in regions I and IV, respectively, of yeast pol δ (Fig. 1). Sequence analysis of PCR-1 demonstrated that it contained an open reading frame possessing extensive similarity to yeast pol δ (ca. 64% identity) and to human

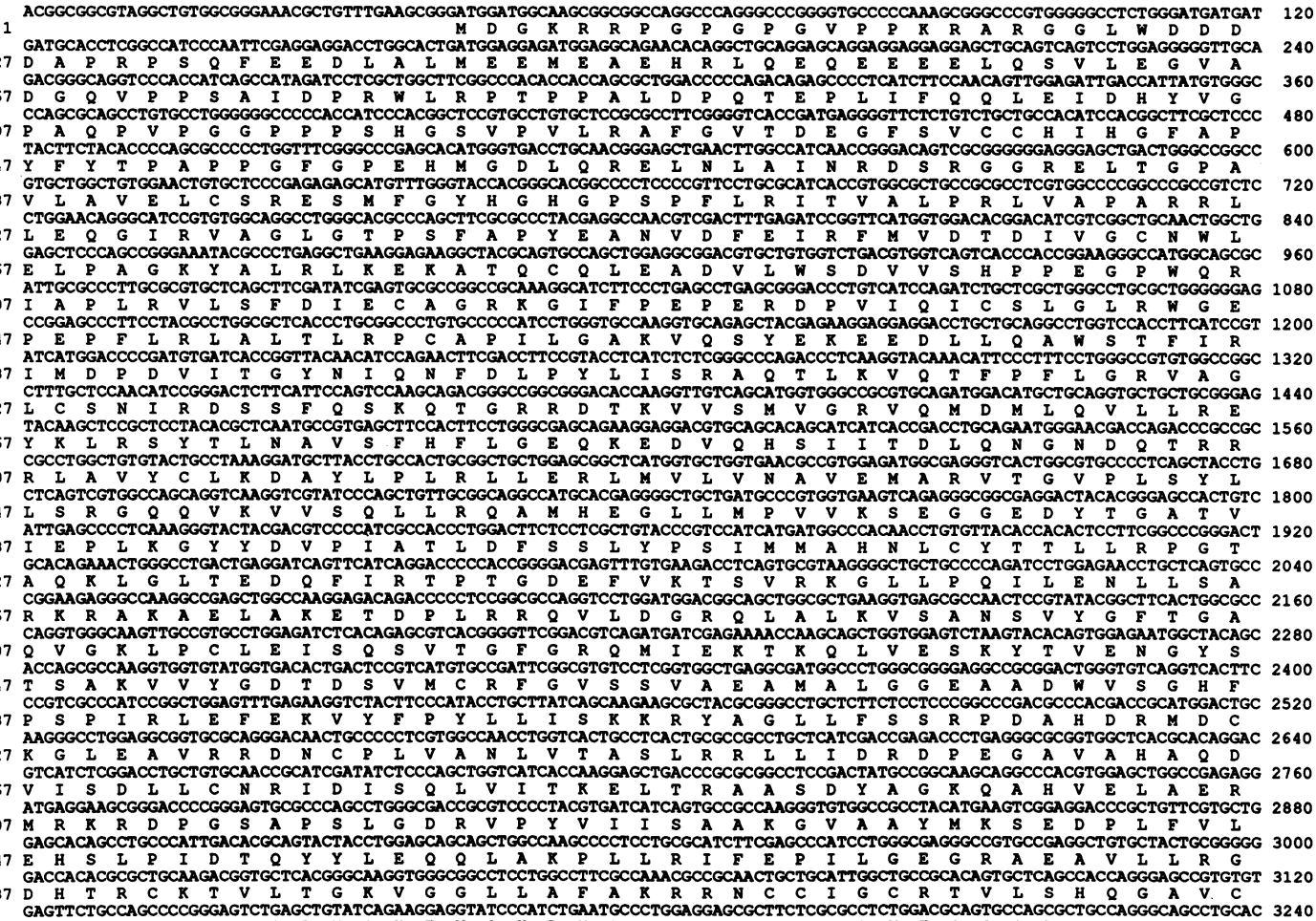

Fig. 2. The nucleotide sequence and deduced amino acid sequence of the cDNA for the catalytic subunit of human pol δ. The diagram shows the nucleotide sequence of the cDNA and the deduced amino acid sequence. The sequence has been submitted to the Genbank data base under the accession number M81735. The deduced amino acid sequence of the fibroblast cDNA clone, HFgt11-1, differed from that of of HLgt10-2 in two positions, in that residues 119 and 173 were found to be arginine and serine, respectively.

pol α (ca. 40% identity). The open reading frame could be aligned from amino acid residues 394 to 764 of yeast pol δ and contained conserved regions IV, II, VI, III and I in correct order and spatial separation (Fig. 1). Parenthetically, from among the first PCR products examined, a clone corresponding to nucleotides 2593−3027 of human pol α (25) using primers based on sequences in regions I and II was also obtained.

The cDNA sequence which encoded the complete primary sequence for pol δ was isolated as follows. Attempts to complete the cDNA sequence in the 5′ direction by PCR primer extension methods from several different human cDNA libraries were only partially successful, possibly due to the high GC content of the cDNA. Only several short PCR fragments were obtained, of which two (PCR-2 and PCR-3) are shown in Fig. 1. Conventional plaque hybridization screening of human cDNA libraries using the PCR-1 clone as a probe yielded a number of positives, of which the three most significant are shown in Fig.1. Clone HLgt10-1 was isolated from the HeLa library. Its DNA sequence overlapped PCR-1 and extended to the 3′ end of the mRNA as shown by the presence of a polyA tail. Clone HFgt11-1 was isolated from the human fibroblast library. Its DNA sequence overlapped HLgt 10-1 and extended almost to the 5′ end of the cDNA. The third cDNA clone, HLgt10-2, was isolated from the HeLa cDNA library using PCR-3 as the probe. The cDNA insert in HLgt10-2 completely overlapped both HLgt10-1 and HFgt11-1 and further extended the cDNA sequence in the 5′ direction (Fig.1). This harbored an open reading frame of 1043 amino acid residues, but did not contain the initiation codon. The 5′ end of the cDNA (PCR-4, Fig 1)was isolated by the 'RACE' PCR primer extension method (30) using linker-primers and three antisense primers located at the 5′ end of HLgt10-2.

The cDNA consisted of 3502 nucleotides, including a polyA tail of 68 nts (Fig. 2). The open reading frame encoded a protein of 1107 amino acid residues with a calculated molecular mass of 124 kDa. This is consistent with the observed relative molecular weight of 125 kDa for the human placental pol δ catalytic polypeptide (8). Analysis of the codon usage revealed that this was extremely biased to G or C in the third position; the overall GC content of the cDNA was 65%. The 3′ noncoding region consisted of 66 nts and contained a polyadenylation signal (AATAAA) 26 residues upstream from the polyA tail. The sequence (AGCGGG) immediately before the methionine start codon is consistent with the Kozak criteria for a translation initiation site (50).

Identification of the cDNA by immunochemical methods

The amount of pol δ protein that can be prepared is very small (8), and attempts to obtain protein sequence data were unsuccessful even with pooled enzyme preparations. An alternative approach employing immunochemical methods was therefore used to confirm the identity of the cloned cDNA. The peptide DLEDQEQLLRRFGPPGPEAW, corresponding to the C-terminal 20 residues of the open reading frame encoded in the cDNA was synthesized, and a polyclonal antibody to this peptide was raised in mice (Materials and Methods). The antisera was purified by affinity chromatography and tested for its ability to recognize human pol δ by immunoblotting. Purified pol δ was subjected to HPLC on a Mono-S column and the active fractions were immunoblotted with the polyclonal antibody. A 125 kDa polypeptide with intensities which corresponded to the peak of pol δ enzyme activity was detected (Fig. 3, left inset). As a control (Fig. 3, right inset) the peak fraction of pol δ activity was immunoblotted using a specific monoclonal antibody against human pol δ (8). This antibody also immunoblotted a 125 kDa polypeptide. Also, a monoclonal antibody raised against the same C-terminal peptide specifically immunoblotted pol δ but did not immunoblot either human pol α or pol ε (Fig 4A).

Since one of the cDNA clones that we isolated (HFgt11-1) originated from a human fibroblast cDNA library constructed in λgt11 expression phage, we conducted experiments to determine if it produced a fusion protein which was immunoreactive with a monoclonal antibody specific for human

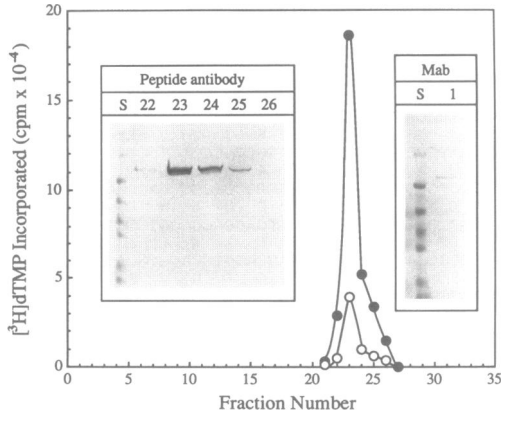

Fig. 3. Immunochemical reactivity of a polyclonal antibody against the C-terminal peptide of pol δ. Purified DNA polymerase δ (8) was chromatographed on a Waters 650 Instrument using a Pharmacia HR 5/5 Mono-S column. Elution was performed using a gradient of 0 to 600 mM KCl and fractions of 30 fractions were collected and assayed in the presence (●) and absence (○) of PCNA. The peak fractions were Western blotted (8) using a polyclonal antibody against the C-terminal peptide (left inset). The peak fraction was also immunoblotted using monoclonal antibody 'A' (right inset), which is specific for pol δ and does not react with pol α or ε (8).

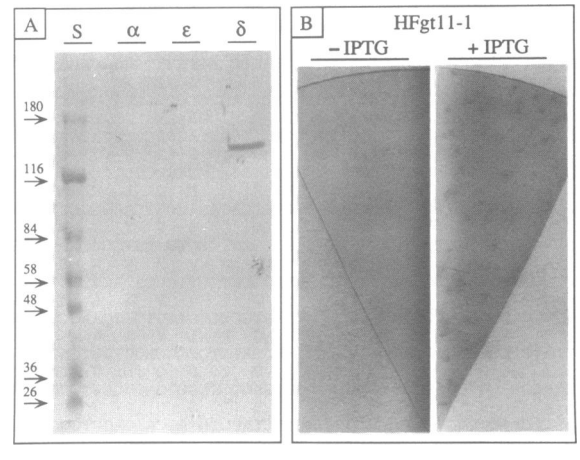

Fig. 4. Western blotting of human pol δ by a monoclonal antibody against the C-terminal peptide and immunoreactivity of the fusion protein expressed by HFgt11-1. *Panel A.* A monoclonal antibody against the C-terminal peptide of pol δ was used to immunoblot purified DNA polymerase δ, α and ε. Lane 'S', prestained protein standards; Lanes 'α', 'ε', and 'δ', purified human placental DNA polymerases α, ε, and δ (5 units of each). *Panel B.* The positive cDNA expression phage HFgt11-1 was plated. Nitrocellulose lifts were performed and immunochemically stained with monoclonal antibody 'A' which is specific for pol δ (8). A segment of the stained filter of uninduced phage (-IPTG) is shown on the left and a segment of the filter with IPTG induction is shown on the right.

pol δ (i.e. assuming that the cDNA was fused in-frame with the beta-galactosidase protein). This appeared to be the case, since plaques of the phage showed positive immunochemical staining after induction with IPTG (Fig. 4B), providing further evidence for the identification of the cDNA. No reaction was observed with control phage, or when a control monoclonal antibody (P3), a monoclonal antibody against human pol α, or a polyclonal antibody against pol ε were used (not shown).

Northern blotting of human mRNA

The result of Northern blot analysis of mRNA isolated from human IMR90 fibroblasts using the HLgt10-2 cDNA as a probe is shown in Fig. 5. A single hybridizing band of about 3.3−3.4 kb was detected. Northern blotting of the same mRNA with the PCR clone to human pol α gave a single band of 5.8 kb (not shown) consistent with the reported size of this mRNA (25).

Human pol δ is closely related to its yeast homologs and the herpes virus DNA polymerases

The deduced amino acid sequence of human pol δ was compared with those of a number of other DNA polymerases (Materials and Methods). Comparison of human pol δ with *S. cerevisiae* pol δ, and with the recently cloned *S. pombe* pol δ (46) revealed a very close relationship. *S. cerevisiae* and *S. pombe* delta polymerases (27, 46) are also very similar in size to human pol δ (1097 and 1084 residues, respectively). Using the ALIGN program, the similarities (% identity = identical residues/number of possible matches × 100) of human pol δ to *S. cerevisiae* and *S. pombe* pol δ were found to be 48% and 54%, respectively (Table I). Similarity between the two yeast pol δ sequences was 54%. By comparison, yeast and human pol α have a similarity of 28% when aligned by the same method. Pairwise alignments of the human pol δ with the herpes DNA polymerases gave% similarity scores of 30−33% (Table I). A comparison of the similarities of the N-termini, core and C-terminal regions is also shown in Table I. This was done because previous findings have shown that while the core region is strongly conserved, there is considerable variability in the N- and C-terminal regions of the DNA polymerases in this family (25). While the delta and herpes DNA polymerases show conservation in all three regions, REV3 and pol α are dissimilar in the N-terminus (Table I). A multiple sequence alignment was constructed for the delta and herpes DNA polymerases and the results are shown separately in Figs. 6, 7 and 8 for the N-termini, the core regions and the C-termini, respectively. In the alignments shown, the residues have been marked to show relationships with the delta polymerases. The close similarity of the three delta polymerases, as well as their similarity to the herpes polymerases, are clearly evident in these alignments (Figs. 6,7,8). These results establish that the cDNA that we have cloned encodes a human DNA polymerase whose primary structure identifies it as a delta polymerase, and also establishes its relationship to the human herpes DNA polymerases. Specific aspects of these alignments are described below.

Similarities of pol δ and pol ε to the herpes DNA polymerases at the extreme N-terminal regions. The alignments of the N-termini of the delta and herpes DNA polymerases (Fig. 6) show that they share significant similarities (25−36%, Table I). It was noted that pol ε showed similarities to CMV and HSV at their extreme N-termini and also between regions N2 and N3 (residues 176−207, Fig.6). HSV, VZV and CMV DNA polymerases have the start sequence M-x_n-S-G-G. Within the human pol δ nucleotide sequence there is a second ATG located one nucleotide downstream from the initiation codon (Fig. 2). The alternate reading frame starting from this ATG encodes the sequence **MASGGQAQGPGCPQSGPVGASGMM**, having the identities shown in bold with CMV and HSV (cf. Fig. 6). This raises the possibility that the extreme N-terminal sequences of either pol δ or the herpes polymerases may have arisen through mechanisms involving one or more frame shift mutations during the course of evolution. (This was not due to a DNA sequencing error as the 5′ end of the cDNA was resequenced several times.)

A highly conserved glycine repeat motif in the N-termini of the delta and herpes polymerases. There are several conserved motifs that are apparent within the N-termini. This includes the motif G-x_4-G-xx-V-x-V-x-V-x-G-x_3-YFY (G-x_4-G-x_8-G-x_3-YFY) in the region designated as N2. Part of this motif had previously

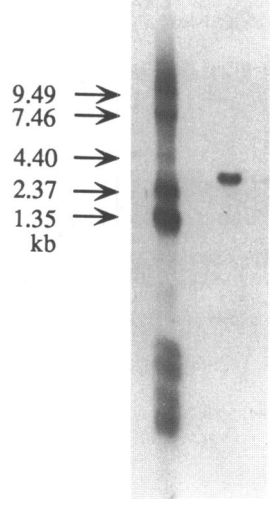

Fig. 5. Northern blotting of human mRNA. Total RNA (30 μg) isolated from human diploid lung fibroblasts IMR90 was run on agarose gels and probed using ^{32}P-labeled HLgt10-2 DNA (Materials and Methods). The left lane shows the ^{32}P labeled RNA standards (Bethesda Research Laboratories).

Table I. Similarities between human DNA pol δ and other eukaryotic DNA polymerases

DNA Polymerase	Overall	N-terminus	Core	C-terminus
S.pombe pol δ	54	36	63	59
S.cerevisiae pol δ	48	31	64	43
EBV	33	32	40	25
CMV	31	29	37	18
HSV	30	28	36	24
VZV	30	25	36	26
REV3	–	–	33	22
Human pol α	–	–	30	28
S.cerevisiae pol α	–	–	30	27
S.cerevisiae pol ε	–	18	26	–

Alignments were performed by the ALIGN program using the mutational data matrix (250 PAMS), bias = 6, penalty = 6. Regions defined as the N-termini, core and C-termini are as given in Figs. 6−8. Similarities were calculated as a percentage of the number of identities per number of possible matches for the given alignments. (Alignments in the regions marked by dashes were not performed because of low degrees of similarity or major differences in length.)

been identified in the herpes DNA polymerases as the motif V-x-V-x-V-x-G-x_3-YFY (51) . Although its potential functional role, if any, is unknown, it is of interest that several motifs involving glycine repeats have been implicated in nucleotide binding. This includes the motif G-x_4-G-K which has been found in a variety of proteins that include ATPases, DNA binding proteins, adenylate kinase, GTP binding proteins (52–55), and the motif G-x-G-x_3-G in cAMP-dependent protein kinase (56). Region N2 was also identified in yeast pol ϵ as well as REV3, which otherwise displayed little similarity to the N-termini of the delta and herpes polymerases.

The 3′ to 5′ exonuclease sites. Three 3′ to 5′ exonuclease sites (Exo I, II and III) have been proposed for the eukaryotic family of polymerases (57) on the basis of alignments with the exonuclease catalytic sites of the E. coli pol I family. The location of all three sites had been assigned to the core (see Fig. 7), with Exo I and Exo II being located approximately in the two halves of region IV (57). Exo I possesses two of the four carboxylate residues (D355 and E357) functionally involved in the 3′ to 5′ exonuclease activity of E. coli Pol I (58, 59). In the alignments shown in Fig. 6, region N5 in the N-terminus harbors the highly conserved sequence, DIEC-x_n-FP which is also conserved in

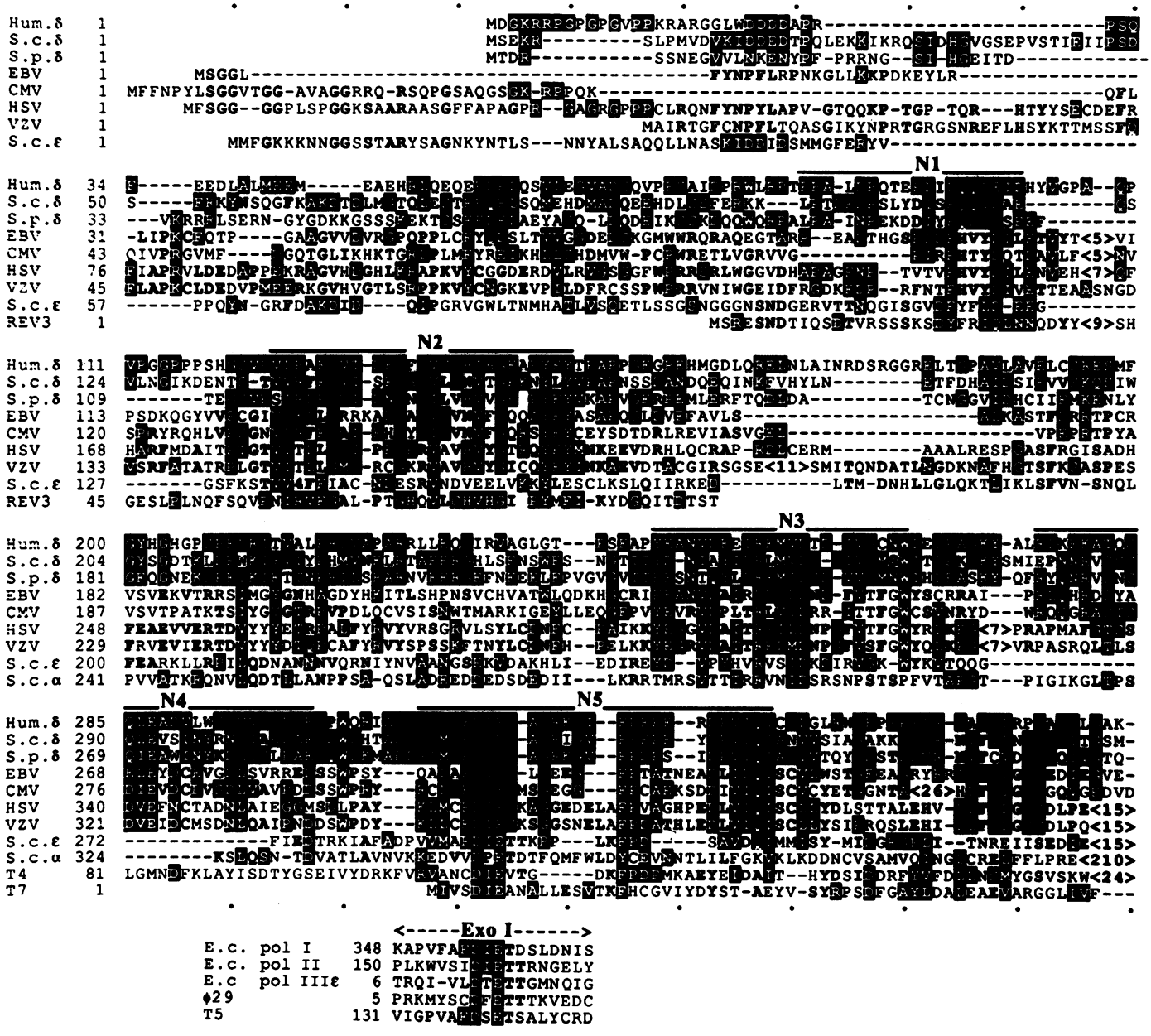

Fig. 6. Alignment of the N-terminus of human pol δ with other eukaryotic and viral DNA polymerases. Sequence alignments were performed as described in 'Materials and Methods' against the N-terminal 367 residues of human pol δ. (Abbreviations: Hum., human; S.c., *S. cerevisiae* ; S.p., *S. pombe* ; EBV, Epstein Barr virus; CMV, cytomegalovirus; VZV,varicella zoster virus; HSV, herpes simplex virus type 1.) Residues identical to those in human pol δ are in were first boxed; conservative replacements with these residues (M, I, L, V; S, T; P, A, G; Y, F; R, K; D, E, N, Q) were then boxed. Other identities are shown in bold, unboxed. Below the alignment are shown some of the sequences aligned in the Exo I site of the 3′ to 5′ exonuclease region of the prokaryotic family of DNA polymerases, taken from ref. 57. We have marked 5 regions, labeled N1 to N5, where there is significant identity between the delta and herpes DNA polymerases.

yeast pol ε, bacteriophage T4 and T7 DNA polymerases. In the case of T7, the sequence DIEA had been aligned previously with the 'Exo I' 3′ to 5′ exonuclease site (57). It was observed that the Exo I site of the procaryotes could equally be placed in the context of an alignment with the N5 region (Fig. 6) . In this case, there is complete conservation of the aspartate and glutamate residues, which is not the case in the previous assignment of this region to Region IV of the core (57). Similar conclusions were reached in recent studies of yeast pol δ and pol ε, in which site-directed mutagenesis of the acidic residues in this motif in the region designated as N5 (D321 and E323, for pol δ; D290 and E292 for pol ε) was found to exert both functional and phenotypical consequences consistent with their having a role in 3′ to 5′ exonuclease catalysis (60, 61). This relocation of the proposed Exo I site also resolves an apparent contradiction when mutagenesis of the T4 polymerase residues asp-189 and glu-191, in the earlier alignment of Bernad et al. (57), failed to affect the 3′ to 5′ exonuclease activity (62).

Exo II is proposed to contain the functional homolog of carboxylate residue corresponding to D424 of E. coli pol I (57). Within the group of delta and herpes polymerases this glutamate residue is contained in a highly conserved motif, **GYN-x_3-FD** (Fig. 7). Mutagenesis of D407 of *S. cerevisiae* pol δ in this motif has recently been shown to result in selective loss of its 3′ to 5′ exonuclease activity (60). The third exonuclease site, Exo III, is proposed to contain the residues analogous to Y497 and E501 of the E. coli Pol I catalytic site (57). This site is located at the start of the region B identified in Fig. 7. This region is not as

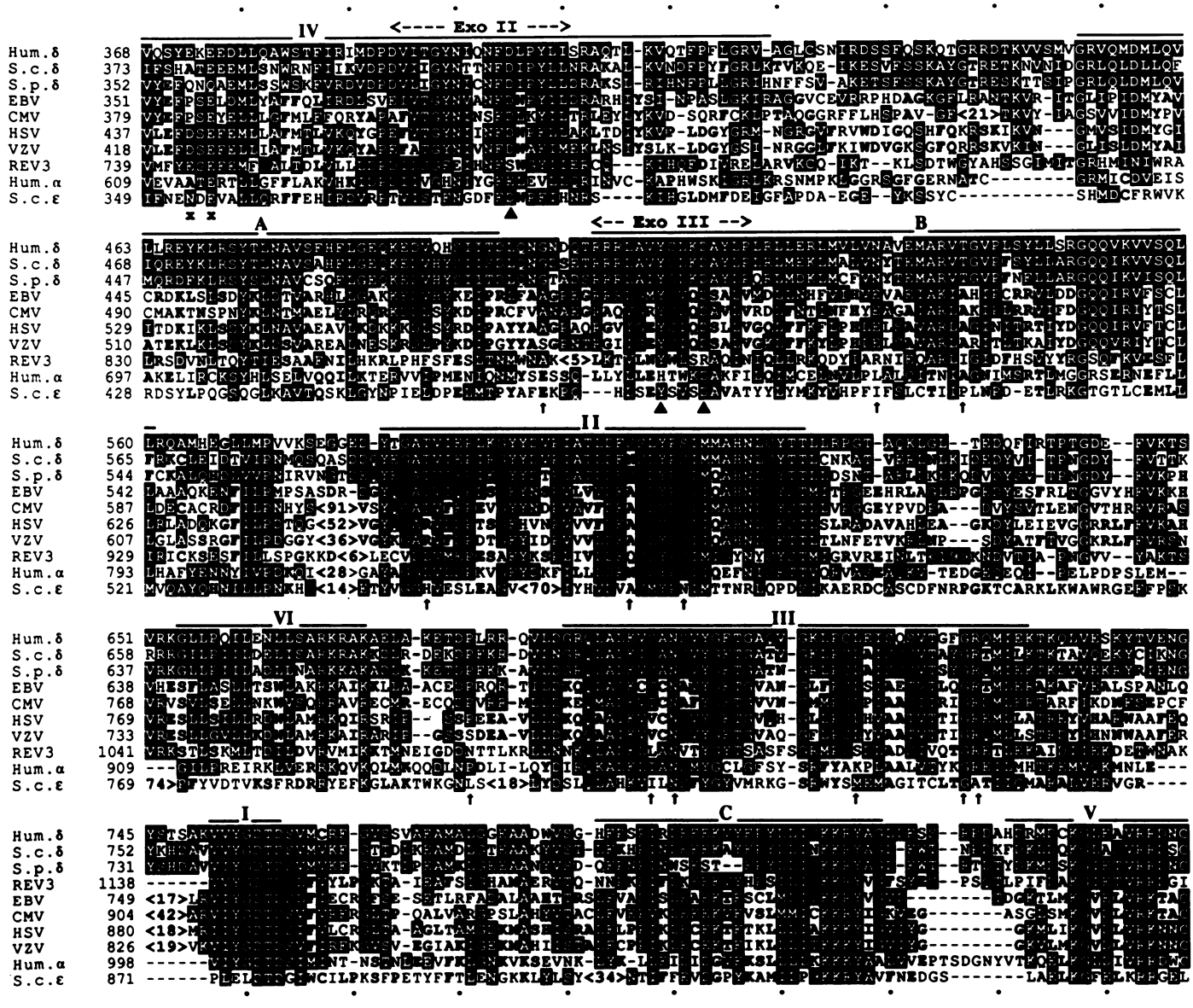

Fig. 7. Alignment of the core region of human pol δ with other eukaryotic and viral DNA polymerases. Sequence alignments were performed as described in Materials and Methods. Key is as in Fig. 6. The core region was defined as that containing conserved regions I-VI as proposed in ref. 25. The symbols below the rows were used as follows: 'x' denotes the position of the aspartate and glutatmate residues in the 'Exo I' region as originally proposed in ref. 57; solid triangles, in order, denote the conserved asparate residue in Exo II, the conserved tyrosine and aspartate residues in Exo III; the arrows denote residues in HSV polymerase where mutations which affect substrate binding (phosphonoacetic acid and nucleotide analog resistance or hypersensitivity) have been found (28, 38, 73).

highly conserved; however, in the delta and herpes polymerases there is absolute conservation of the tyrosine and aspartate residues in the motif **Y**-x_3-**D**-x_2-L.

Identification of a region of similarity of yeast pol I with the delta and herpes polymerases containing a motif corresponding to the Exo I site. Although the N-termini of human and yeast pol α show similarities, they are thought to be quite distinct from those of the other known DNA polymerases. We examined their N-terminal sequences for the presence of the Exo I region, since our alignments suggested that this was not located in the core region. This was done by searching for the motif D-x-E-C/T. As a result, we identified such a sequence, DPET (residues 346−349), in yeast pol α but not in human α. This Exo I motif, like that of yeast pol ε (DIET) more closely resembles that of the prokaryotic DNA polymerases (Fig. 6), where there is considerable variability in the second residue of the motif and where the fourth residue is quite commonly a threonine residue (57). This new finding that yeast pol α has an Exo I motif in the N-terminus is of more than passing interest. It has been reported that yeast pol α has a weak but active 3′ to 5′ exonuclease activity (63), and that Drosophila pol α has a cryptic 3′ to 5′ exonuclease activity (64). The assignment of the DPET sequence of yeast pol α to region N5 is more than pure chance, since we could also align the yeast pol α sequences proximal to the DPET motif. This is shown for residues 241−399 (Fig. 6). Although the alignment is weak, there is a discernable conservation of region N3 as well as N5. This result indicates that previously unsuspected similarities do exist between the N-termini of yeast pol α and the delta/herpes DNA polymerases.

The core regions. Human pol δ exhibits >60% similarity with the two yeast delta polymerases, and between 36−40% identity with the herpes polymerases in the core region (Table I). We have designated two other additional regions, labeled A and B, which are located between IV and II, These are strongly conserved in the three pol δ sequences. The core region of this family of DNA polymerases contains the structural elements responsible for the DNA polymerase activity (dNTP/pyrophosphate binding), as origninally shown by studies of HSV polymerase mutants which exhibit altered sensitivity to nucleotide or pyrophosphate analogs. These are located largely within regions II and III (Fig. 7). Site-directed mutagenesis of the highly conserved motif, YGDTDS (region I) in HSV polymerase has recently demonstrated it to be crucial for polymerase activity (65,66). However, unlike the 3′ to 5′ exonuclease sites, the elements involved in the polymerase catalytic activity are relatively scattered and have not been

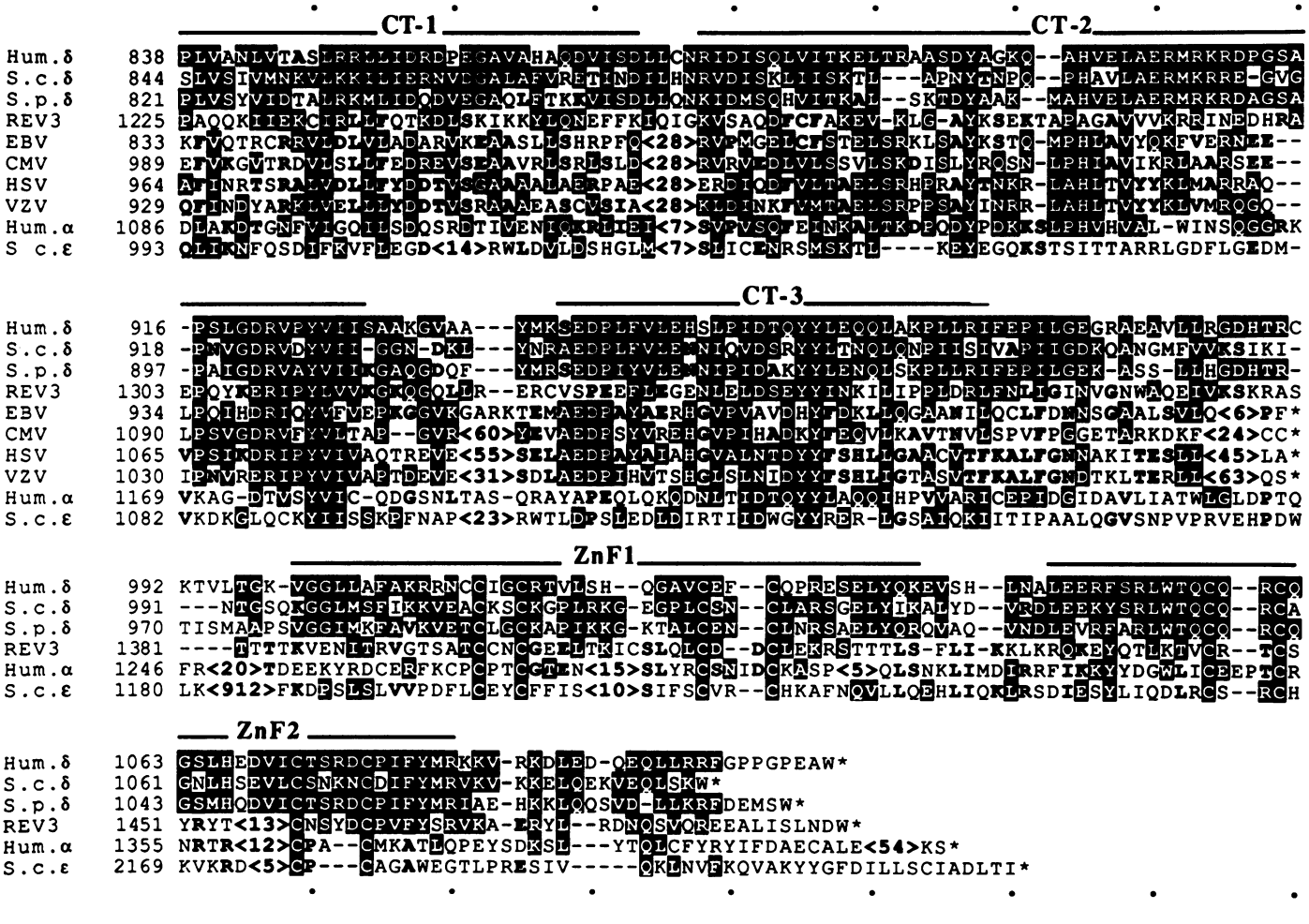

Fig. 8. Alignment of the C-terminus of human pol δ with other eukaryotic and viral DNA polymerases. Key is as in Fig. 6.

mechanistically defined. A highly conserved region between I and V that we have designated as C (Fig.7) is present in all of these polymerases.

The C-termini of the human and yeast delta polymerases are highly conserved. Human pol δ shows a degree of similarity to *S. pombe* pol δ (59%) almost as high as that in the core region (Table I). Within this C-terminal region, we have designated 5 conserved regions, CT1, CT2, CT3, ZnF1 and ZnF2 (Fig. 8). CT1, CT2 and CT3 are conserved in the delta and herpes polymerases, and also in REV3, pol α and ε. The functions of these regions are unknown; however, in the case of HSV, deletion mutagenesis has shown that the C-terminal 227 residues (which would include CT-2 and CT-3) are required for interaction with UL42 (67). These three regions are separated by large insertions in the herpes DNA polymerases, suggesting that these may mark intron-exon boundaries or regions where extensively external looping of the polypeptide chains occur. CT-3 also marks the end of the similarity between the herpes DNA polymerases and the eukaryotic polymerases. Following CT3 is a region containing two putative zinc finger structures, first identified in pol α (25). These are present in the regions designated as ZnF1 and ZnF2. The zinc finger regions of the three delta polymerases are highly conserved and show less conservation with those of pol α, pol ε and REV3. The ZnF2 region in the sequence beginning LEE (residues 1047−1082) is particularly well conserved. Within these 36 residues the human sequence shares 67% identity with yeast pol δ (residues 1045−1080), and 89% identity with *S. pombe* pol δ (residues 1027−1062). ZnF1 and ZnF2 are obvious candidates for the location of the site(s) involved in the interaction of pol δ with PCNA. The observed abilities of yeast and mammalian PCNA and pol δ for heterologous, functional interactions (4, 68), almost certainly requires a highly conserved interaction site in yeast and human pol δ. Moreover, the location of a PCNA binding site proximal to a zinc finger motif with potential DNA binding functions could provide insights into the mechanistic basis for the effects of PCNA on the processivity of pol δ.

Human pol δ message increases in serum-stimulated proliferating cells

The expression of the DNA pol δ message was examined in serum-starved quiescent IMR90 human fibroblast cells that were induced to proliferate by serum stimulation. The pol δ message levels increased dramatically up to a 10-fold level at 30 hours (Fig 9). The enzymatic activity of pol δ was induced in parallel starting at 6 hours, but leveled off at 12 hours, in contrast to the mRNA levels which were still rising at this time. DNA synthesis was increased, starting at 12 hours after serum stimulation, and leveled off in parallel with the pol δ activity. The expression of pol δ mRNA and activity thus follows a pattern consistent with its function as a nuclear DNA replication enzyme.

The human pol δ gene is located on chromosome 19

Southern blot analysis of EcoRI digested DNA from human, mouse and hamster cells, and a panel of human/rodent cell

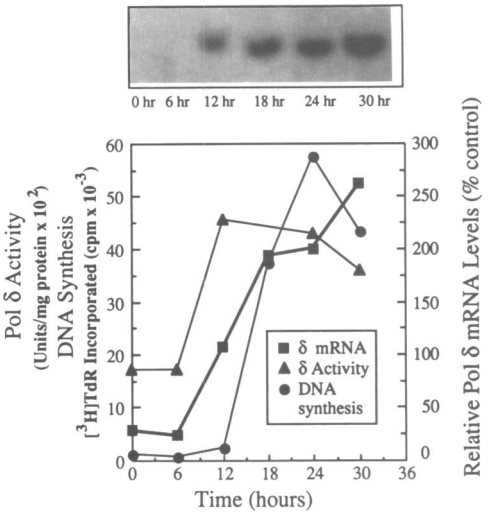

Fig. 9. Expression of pol δ mRNA in serum-restimulated cells. IMR90 cells were serum deprived for 5 days in Earle's minimum essential medium, 0.1 mM nonessential amino acids containing antibiotics and 0.1% fetal calf serum. After 5 days, cells were stimulated by replacement of medium containing 10% fetal bovine serum. Cells were harvested at 6 hr intervals after serum stimulation. Equal amounts of RNA isolated from the cells were run on agarose gels and Northern blotted (Materials and Methods). The relative amounts of mRNA were determined by densitometric scanning of the autoradiograms. These were compared to that of the blot of mRNA from normally growing cells (control value of 100%). DNA polymerase δ activity was determined in the extracts by assaying in the presence and absence of 100 ng of PCNA or 20% DMSO (74, 75). Total DNA synthesis was measured by [^{3}H]thymidine incorporation. The upper panel shows the blots from which the densitometric traces were made.

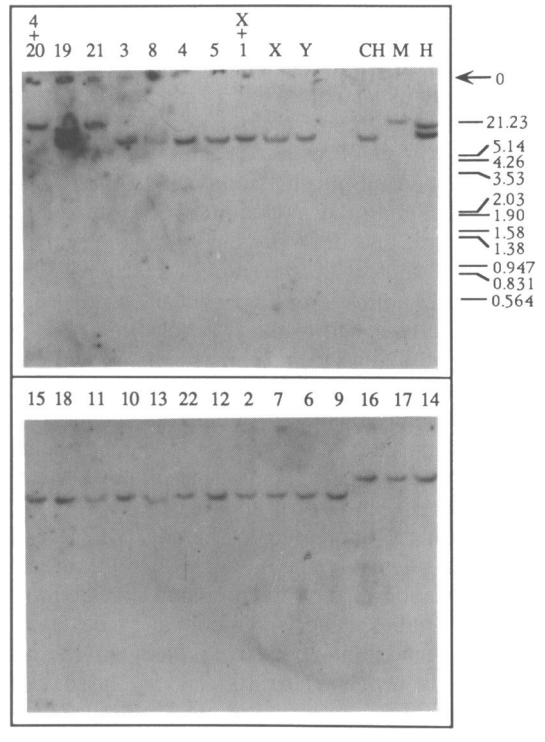

Fig. 10. Determination of the chromosomal localization of the human Pol δ gene. High molecular weight DNAs (10 μg each), isolated from the human/rodent cell hybrids and their parental cell lines (human IMR-91, mouse 3T6 and Chinese hamster RJK88), were digested with EcoRI. The digested DNAs were electrophoresed on 0.7% agarose gels and transferred to GeneScreen Plus nylon filter membranes. These were hybridized with a ^{32}P-labeled cDNA probe from HLgt10−2 and autoradiographed. The human chromosomes present in the hybrids are shown above the lanes. CH, M, and H are the DNAs of the control hamster, mouse and human cells, respectively. Migration of the standard DNAs are shown on the right.

hybrids of defined human chromosomal content was performed (Fig.10). In both mouse and hamster DNA, a single hybridizing EcoRI restriction fragment (about 26 kb and 10 kb, respectively) was detected, indicating the existence of a single gene. However, in human DNA, two hybridizing DNA bands were detected, with approximate sizes of 20 kb and 12 kb, suggesting the size of the human gene to be at least 32 kb. Southern blot analysis of DNAs isolated from the somatic cell hybrids showed that the characteristic two bands observed in human DNA were present only in cell line NA 10449, containing chromosome 19 (Fig. 10). This unambigous result shows that the human pol δ gene is located on chromosome 19. The localization of human δ gene to chromosome 19 is of interest, because of the central position of pol δ in chromosomal DNA synthesis. Its localization may be of potential significance because it represents a potential locus for mutations which involve derangements in DNA synthesis or its regulation.

DISCUSSION

We have used a combination of PCR amplification and screening of cDNA libraries to isolate the cDNA for human pol δ. The positive identification of the cDNA as encoding pol δ is inferred from the following: 1) Western blotting experiments using polyclonal or monoclonal antibodies against the 20-residue C-terminal peptide provide immunochemical evidence for its presence in authentic human pol δ. Examination of the λgt11 cDNA expression clone shows that it behaves in a manner consistent with the expression of a fusion protein containing an epitope recognized by a monoclonal antibody against authentic pol δ. 2) A single hybridizing band of 3.4 kb was observed by Northern blot analysis, consistent with the length of the cDNA. This mRNA is too small to encode either pol ε or pol α, and moreover, it is induced in cells stimulated to proliferate with a time-course paralleling the induction of pol δ activity. 3) The open reading frame encodes a protein with a molecular mass of 124 kDa, consistent with the reported size of the pol δ polypeptide. 4) Examination of the primary sequence shows it to be more closely related to the two yeast (*S. cerevisiae* and *S. pombe*) pol δ proteins than to any of the known eukaryotic DNA polymerases. [In the course of this work, the gene for pol δ from the malarial parasite P. falciparum has been isolated. (R.G. Ridley, personal communication). The open reading frame of this gene encodes a protein which also shows the expected close conservation with human and yeast pol δ.]

The results on the expression of human pol δ message are consistent with its involvement in DNA replication, and is suggestive of an association of its expression with the proliferative state. Previous studies using whole animal tissues have shown that pol δ activity and immunoreactive protein are decreased with development of the neonatal heart (69) and increased during liver regeneration (70). The expression of pol δ message level is quite similar to that reported for pol α under conditions of serum-stimulated proliferation (71). In the latter study, the issue of the whether the increase of message after stimulation of serum-depleted quiescent cells is truly reflective of the situation in normal cell cycling was examined by techniques allowing the separation of cells at distinct phases of the cell cycle. It was found that pol α is constitutively expressed throughout the entire cell cycle and was modestly increased during the S phase. Thus, further examination of the expression of the human pol δ message may be revealing and is currently under investigation. The fact that pol δ mRNA levels clearly can undergo significant changes obviously needs further investigation to confirm whether this is due to de novo synthesis. The determination of its gene structure, particularly of the 5′ flanking region, would be of extreme interest since it seems possible that pol δ, pol α, pol ε, PCNA or other proteins required for DNA replication may share common regulatory mechanisms at the gene level.

The isolation of the cDNA for human pol δ now provides sequence information which has enabled us to analyze its relationships with the other eukaryotic DNA polymerases on a structural basis. Previously, immunochemical studies using specific monoclonal antibodies and polyclonal antibodies had demonstrated that a) human pol δ was distinct from pol α and pol ε and β) that among the panel of antibodies generated from δ there were individual antibodies which were capable of recognizing human pol α, δ and ε, establishing them as an immunochemically related group of proteins (8). This is now confirmed at the structural level by the cloning of the human pol δ. Moreover, during the past 3 years there have been rapid and significant advances in the study of the eukaryotic DNA polymerases, resulting in the cloning of the genes of four *S. cerevisiae* polymerases (pol α, δ, ε, and *MIP1*), a putative polymerase from *S. cerevisiae* (REV3), and the pol δ gene of *S. pombe* (26, 27, 44, 46, 47, 72). The concept that the yeast DNA polymerases are represented by homologous proteins in higher eukaryotes (23) has now been demonstrated at the structural level for yeast and human pol α (25, 26) and in the present work, for human and yeast pol δ.

In spite of the lack of a crystal structure for any of the eukaryotic DNA polymerases, it is encouraging that evidence for functional domains responsible for the 3′ to 5′ exonuclease activity has recently been obtained by the mutagenesis of the carboxylate residues of Exo regions I and II (60, 61). Moreover, these investigations were based on a hypothesis that the functional elements of the catalytic site of E. coli Pol I, the only DNA polymerase for which a crystal structure has been determined to date (58,59), are conserved in the eukaryotic DNA polymerases (57). Thus, the original hypothesis of Bernad et al. (57), which was based on considerations of sequence alignments, is now supported by mutagenesis studies. Since the E. coli Pol I and eukaryotic DNA polymerase families otherwise show little conservation of primary sequence, this suggests that significant conservation of tertiary structure may exist, at least in the case of the 3′ to 5′ exonuclease domains (57).

Previous studies have shown that yeast pol δ is related to the human herpes DNA polymerases (27). This relationship is now confirmed in the case of human pol δ, and clearly suggests their common evolutionary origin. Moreover, it indicates that the information derived from a number of mutational studies of the herpes polymerases have direct implications for understanding potential functional regions of human pol δ. The present determination of the primary sequence of human pol δ, together with those of two other delta polymerases (27, 46), provides a valuable database for assessing the commonly conserved residues with the related herpes polymerases. This may provide insights into their potential structure function relationships. Such comparisons at the present, together with mutational data in the yeast delta and herpes polymerases should allow for more rapid advances in our understanding of the eukaryotic DNA polymerases. Moreover, determination of the crystal structure of any one of the members of this family would be of broad significance.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grant GM31973 to MYWTL and in part by grants from the National Cancer Institute (CA 54323) and the Bremer and Milheim Foundation to LSC. An account of this work was presented at the Cold Spring Harbor Meeting on Eukaryotic DNA Replication in September 1991. We thank Drs A. Sugino, A. Morrison and G. Pignede for copies of their papers in press.

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